UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

NOTICE OF ALLOWANCE AND FEE(S) DUE

21874 7590 02/19/2004

EDWARDS & ANGELL, LLP
P.O. BOX 55874
BOSTON, MA 02205

EXAMINER	
TUNG, JOYCE	
ART UNIT	PAPER NUMBER
1637	

DATE MAILED: 02/19/2004

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/889,379	07/16/2001	Hiroshi Sugiyama	56212 (71526)	2742

TITLE OF INVENTION: DEVELOPMENT OF METHOD FOR SCRENING PHYSIOLOGICALLY ACTIVE PYRROLE IMIDAZOLE DERIVATIVE

APPLN. TYPE	SMALL ENTITY	ISSUE FEE	PUBLICATION FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	NO	$1330	$0	$1330	05/19/2004

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE REFLECTS A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE APPLIED IN THIS APPLICATION. THE PTOL-85B (OR AN EQUIVALENT) MUST BE RETURNED WITHIN THIS PERIOD EVEN IF NO FEE IS DUE OR THE APPLICATION WILL BE REGARDED AS ABANDONED.

HOW TO REPLY TO THIS NOTICE:

I. Review the SMALL ENTITY status shown above.

If the SMALL ENTITY is shown as YES, verify your current SMALL ENTITY status:
A. If the status is the same, pay the TOTAL FEE(S) DUE shown above.

B. If the status is changed, pay the PUBLICATION FEE (if required) and twice the amount of the ISSUE FEE shown above and notify the United States Patent and Trademark Office of the change in status, or

If the SMALL ENTITY is shown as NO:

A. Pay TOTAL FEE(S) DUE shown above, or

B. If applicant claimed SMALL ENTITY status before, or is now claiming SMALL ENTITY status, check the box below and enclose the PUBLICATION FEE and 1/2 the ISSUE FEE shown above.

☐ Applicant claims SMALL ENTITY status.
See 37 CFR 1.27.

II. PART B - FEE(S) TRANSMITTAL should be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). Even if the fee(s) have already been paid, Part B - Fee(s) Transmittal should be completed and returned. If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed and an extra copy of the form should be submitted.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

IMPORTANT REMINDER: Utility patents issuing on applications filed on or after Dec. 12, 1980 may require payment of maintenance fees. It is patentee's responsibility to ensure timely payment of maintenance fees when due.

PTOL-85 (Rev. 11/03) Approved for use through 04/30/2004.

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), to: Mail **Mail Stop ISSUE FEE**
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

or Fax **(703) 746-4000**

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 4 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Legibly mark-up with any corrections or use Block 1)

21874 7590 02/19/2004

EDWARDS & ANGELL, LLP
P.O. BOX 55874
BOSTON, MA 02205

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being facsimile transmitted to the USPTO, on the date indicated below.

(Depositor's name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/889,379	07/16/2001	Hiroshi Sugiyama	56212 (71526)	2742

TITLE OF INVENTION: DEVELOPMENT OF METHOD FOR SCRENING PHYSIOLOGICALLY ACTIVE PYRROLE IMIDAZOLE DERIVATIVE

APPLN. TYPE	SMALL ENTITY	ISSUE FEE	PUBLICATION FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	NO	$1330	$0	$1330	05/19/2004

EXAMINER	ART UNIT	CLASS-SUBCLASS
TUNG, JOYCE	1637	435-006000

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

❑ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.

❑ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list (1) the names of up to 3 registered patent attorneys or agents OR, alternatively, (2) the name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1 _____

2 _____

3 _____

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. Inclusion of assignee data is only appropriate when an assignment has been previously submitted to the USPTO or is being submitted under separate cover. Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE (B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent); ❑ individual ❑ corporation or other private group entity ❑ government

4a. The following fee(s) are enclosed:

❑ Issue Fee

❑ Publication Fee

❑ Advance Order - # of Copies _____

4b. Payment of Fee(s):

❑ A check in the amount of the fee(s) is enclosed.

❑ Payment by credit card. Form PTO-2038 is attached.

❑ The Director is hereby authorized by charge the required fee(s), or credit any overpayment, to Deposit Account Number _____ (enclose an extra copy of this form).

Director for Patents is requested to apply the Issue Fee and Publication Fee (if any) or to re-apply any previously paid issue fee to the application identified above.

(Authorized Signature) (Date)

NOTE: The Issue Fee and Publication Fee (if required) will not be accepted from anyone other than the applicant; a registered attorney or agent; or the assignee or other party in interest as shown by the records of the United States Patent and Trademark Office.

TRANSMIT THIS FORM WITH FEE(S)

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/889,379	07/16/2001	Hiroshi Sugiyama	56212 (71526)	2742

21874 7590 02/19/2004

EDWARDS & ANGELL, LLP
P.O. BOX 55874
BOSTON, MA 02205

EXAMINER	
TUNG, JOYCE	
ART UNIT	**PAPER NUMBER**
1637	

DATE MAILED: 02/19/2004

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(application filed on or after May 29, 2000)

The Patent Term Adjustment to date is 0 day(s). If the issue fee is paid on the date that is three months after the mailing date of this notice and the patent issues on the Tuesday before the date that is 28 weeks (six and a half months) after the mailing date of this notice, the Patent Term Adjustment will be 0 day(s).

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) system (http://pair.uspto.gov).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (703) 305-1383. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at (703) 305-8283.

PTOL-85 (Rev. 11/03) Approved for use through 04/30/2004.

Notice of Allowability	Application No. 09/889,379	Applicant(s) SUGIYAMA ET AL.	
	Examiner Joyce Tung	Art Unit 1637	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☒ This communication is responsive to *11/26/2003*.

2. ☒ The allowed claim(s) is/are *claims 1-5, 19, 22-26 and 28-29 (final claims are claims 1-13)*.

3. ☒ The drawings filed on *16 July 2001* are accepted by the Examiner.

4. ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
   a) ☒ All b) ☐ Some* c) ☐ None of the:
      1. ☒ Certified copies of the priority documents have been received.
      2. ☐ Certified copies of the priority documents have been received in Application No. _____ .
      3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

   * Certified copies not received: _____.

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

5. ☐ A SUBSTITUTE OATH OR DECLARATION must be submitted. Note the attached EXAMINER'S AMENDMENT or NOTICE OF INFORMAL PATENT APPLICATION (PTO-152) which gives reason(s) why the oath or declaration is deficient.

6. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
   (a) ☐ including changes required by the Notice of Draftsperson's Patent Drawing Review (PTO-948) attached
      1) ☐ hereto or 2) ☐ to Paper No./Mail Date _____.
   (b) ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____.

   Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).

7. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☐ Notice of References Cited (PTO-892)
2. ☐ Notice of Draftperson's Patent Drawing Review (PTO-948)
3. ☑ Information Disclosure Statements (PTO-1449 or PTO/SB/08), Paper No./Mail Date 6/23/03
4. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material
5. ☐ Notice of Informal Patent Application (PTO-152)
6. ☒ Interview Summary (PTO-413), Paper No./Mail Date *2/5/2004* .
7. ☒ Examiner's Amendment/Comment
8. ☒ Examiner's Statement of Reasons for Allowance
9. ☐ Other _____.

EXAMINER'S AMENDMENT

Following entry the amendment filed 11/26/2003, the claims 1-5, 18-19, 22-26 and 28-29

1. An examiner's amendment to the record appears below. Should the changes and/or additions be unacceptable to applicant, an amendment may be filed as provided by 37 CFR 1.312. To ensure consideration of such an amendment, it MUST be submitted no later than the payment of the issue fee.

Authorization for this examiner's amendment was given in a telephone interview with John B. Alexander on 2/5/2004.

2. The application has been amended as follows:

Cancel claim 18.

In claim 1, line 6, after "wherein m is 2 to 30, and", delete "X_1 to Xm are, each independently, -CH= or –N=" and substitute therefore – each occurrence of Xm is independently selected from –CH= or –N= --.

In claim 2, line 7, after "the general formula", delete – (I) --.

In claim 19, line 1, delete "The" and substitute –A --; after "kit", delete "according to claim 18" and substitute -- suitable for use in the method of detecting or identifying an action of a chemical species to a substance containing DNA or RNA, wherein the kit --; after "comprising", delete "a" and substitute -- the --.

In claim 19, line 2; delete "which can bind to a base sequence of DNA".

In claim 19, line 7, after "wherein m is 2 to 30, and", delete "X_1 to Xm are, each independently, -CH= or –N=" and substitute therefore – each occurrence of Xm is independently selected from –CH= or –N= --.

In claim 22, line 2, after "an action of", delete "a" and substitute therefore – said --, and after "chemical species", delete "A".

3. The following is an examiner's statement of reasons for allowance:

Concerning claims 1-5, 18-19, 22-26 and 28-29, no prior art has been found teaching or suggesting the chemical species as represented in claim 1, 19 and 28-29 used in the method for detecting or identifying an action of the chemical species to a substance containing DNA or RNA and the kit containing the chemical species as represented in claim19.

The closest prior art is the reference of Asai et al. (J. Am. Chem. Soc., 1994, Vol. 116, pg. 4171-4177). Asai et al. disclose a novel property of Duocarmycin and its analogues for covalent reaction with DNA. The chemical compound (See pg. 4174, the Abstract and fig. 1) used in the method of Asai et al. is not the same chemical species as represented in claim 1.

Any comments considered necessary by applicant must be submitted no later than the payment of the issue fee and, to avoid processing delays, should preferably accompany the issue fee. Such submissions should be clearly labeled "Comments on Statement of Reasons for Allowance."

4. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Joyce Tung whose telephone number is (571) 272-0790. The examiner can normally be reached on Monday - Friday, 8:30-5:00.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Gary Benzion can be reached on (571) 272-0782. The fax phone number for the organization where this application or proceeding is assigned is 703-872-9306.

5. Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

Joyce Tung J.T
February 6, 2004

ETHAN WHISENANT
PRIMARY EXAMINER

J. Am. Chem. Soc. 1994, 116, 4171–4177 file copy

A Novel Property of Duocarmycin and Its Analogues for Covalent Reaction with DNA

Akira Asai, Satoru Nagamura, and Hiromitsu Saito*,†

Contribution from the Tokyo Research Laboratories, Kyowa Hakko Kogyo Co., Ltd. 3-6-6, Asahi-machi, Machida-shi, Tokyo, 194 Japan

Received December 6, 1993•

Abstract: For understanding the mechanism of action of antitumor agents and designing new drugs, the DNA alkylating property of duocarmycin (DUM) and its analogues was examined. The thermal depurination products of calf thymus DNA covalently bonded to DUMA were revealed to be not only the DUMA–N3 adenine adduct but also unexpectedly the DUMA–N3 guanine adduct. In addition DUMSA and synthetic analogues, **1** and **2** with higher solvolytic stability, reacted more selectively with N3 adenine than DUMA did. The correlation between electrophilicity of the cyclopropane subunit in the molecule and selectivity to adenine was observed. KW-2189, the synthetic derivative of **1** which has improved *in vivo* antitumor activity, was designed as a prodrug requiring enzymatic hydrolysis of the carbamoyl moiety, followed by regeneration of **1**. Surprisingly we discovered that KW-2189 itself alkylated DNA covalently without release of the carbamoyl moiety. For the mechanism of DNA alkylation by KW-2189, a novel alkylating reaction via the formation of an iminium intermediate **18** without loss of the carbamoyl moiety was proposed.

A new class of antitumor antibiotics produced by *Streptomyces sp.*, including duocarmycin (DUM) A,[1-3] B_1,[4] B_2,[4] C_1,[3] C_2,[2,3] and SA[5-7] possess exceptionally potent cytotoxicity. DUMA, C_1, and C_2 have been reported to derive their cytotoxicity through a sequence-selective minor groove alkylation of double-stranded DNA which mediates, as in the case of the antitumor antibiotic CC-1065, N3 adenine covalent adduct formation.[8-11] In the course of our investigation of the thermal depurination products of DNA covalently bonded to DUMA, we obtained not only the DUMA–N3 adenine adduct but also unexpectedly the DUMA–N3 guanine adduct. Herein we report the isolation and full characterization of the DUMA–guanine covalent adduct and adenine adducts of synthetic derivatives. In addition we estimated the reactivity of DUM and synthetic derivatives with DNA by HPLC. The correlation between electrophilicity of cyclopropane and selectivity to adenine was observed. KW-2189 is the synthetic derivative of **1** with improved *in vivo* antitumor activity. Based on its superior solubility and its improved *in vivo* antitumor activity, KW-2189 has been selected for use in clinical trials. Surprisingly we discovered that KW-2189 itself alkylated DNA covalently without release of the carbamoyl moiety.

Stability of Cyclopropane Subunit. The potent cytotoxicity of DUMA and its related compound, CC-1065, both of which contain the spirocyclopropylhexadienone moiety as a common pharmacophore, is thought to be attributable to their DNA alkylating ability. It has been reported that these agents bind to the AT-rich minor groove of DNA and then receive adenine N_3 addition to the unsubstituted cyclopropane carbon, generating the drug DNA covalent adduct.[8-11] To investigate the reactivity of the cyclopropane subunit of DUMA, DUMSA, and analogues **1** and **2**, we evaluated the chemical stability of these drugs under aqueous neutral conditions by using reverse-phase HPLC analysis. Figure

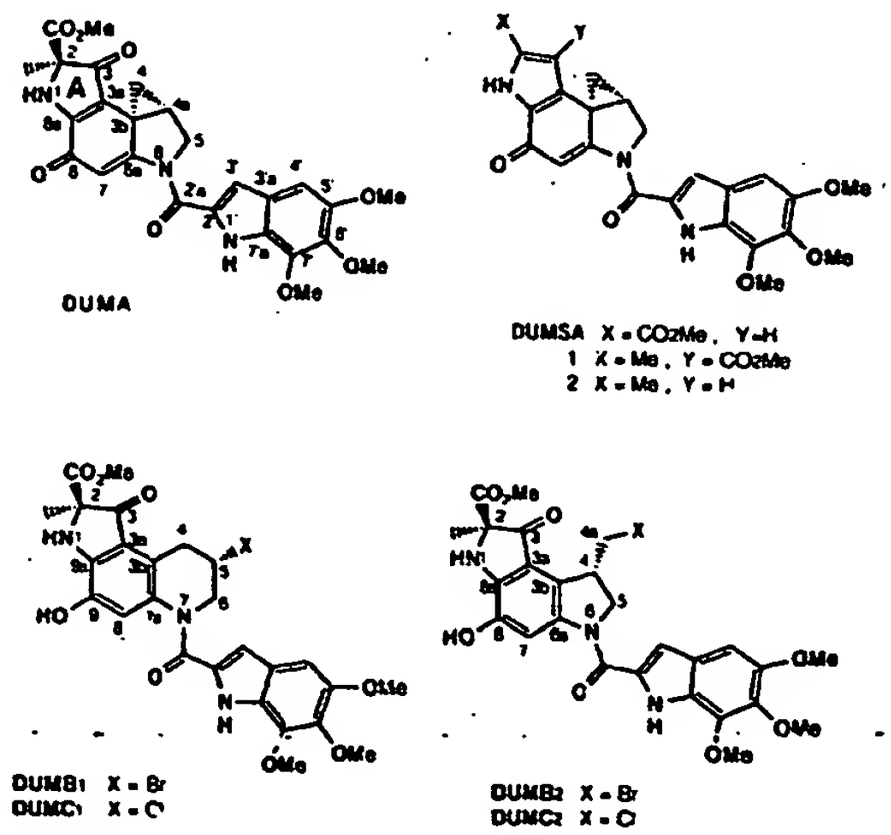


Figure 1. Structures of ducarmycins and analogues described in this study.

† Present address: Pharmaceutical Research Laboratories, Kyowa Hakko Kogyo Co., Ltd., Shimotogari 1188, Nagaizumicho, Sunto-gun, Shizuoka 411, Japan.

• Abstract published in *Advance ACS Abstracts*, April 15, 1994.

(1) Takahashi, I.; Takahashi, K.; Ichimura, M.; Morimoto, M.; Asano, K.; Kawamoto, I.; Tomita, F.; Nakano, H. *J. Antibiot.* **1988**, *41*, 1915.
(2) Yasuzawa, T.; Iida, T.; Muroi, K.; Ichimura, M.; Takahashi, K.; Sano, H. *Chem. Pharm. Bull.* **1988**, *36*, 3728.
(3) Ichimura, M.; Muroi, K.; Asano, K.; Kawamoto, I.; Tomita, F.; Morimoto, M.; Nakano, H. *J. Antibiot.* **1988**, *41*, 1285.
(4) Ogawa, T.; Ichimura, M.; Katsumata, S.; Morimoto, M.; Takahashi, K. *J. Antibiot.* **1989**, *42*, 1299.
(5) Ichimura, M.; Takahashi, K.; Kobayashi, E.; Kawamoto, I.; Yasuzawa, T.; Nakano, H. *J. Antibiot.* **1990**, *43*, 1037.
(6) Ichimura, M.; Ogawa, T.; Katsumata, S.; Takahashi, K.; Takahashi, I.; Nakano, H. *J. Antibiot.* **1991**, *44*, 1045.
(7) Yasuzawa, T.; Saitoh, Y.; Ichimura, M.; Takahashi, I.; Nakano, H. *J. Antibiot.* **1991**, *44*, 445.
(8) Sugiyama, H.; Hosoda, M.; Saito, I.; Asai, A.; Saito, H. *Tetrahedron Lett.* **1990**, *31*, 7197.
(9) Boger, D. L.; Ishizaki, T.; Zarrinmayeh, H.; Kitos, P. A.; Suntornwat, O. *J. Org. Chem.* **1990**, *55*, 4499.
(10) Boger, D. L.; Ishizaki, T.; Zarrinmayeh, H.; Munk, S. A.; Kotos, P. A.; Suntornwat, O. *J. Am. Chem. Soc.* **1990**, *112*, 8961.
(11) Boger, D. L.; Ishizaki, T.; Zarrinmayeh, H. *J. Am. Chem. Soc.* **1991**, *113*, 6645.

0002-7863/94/1516-4171$04.50/0

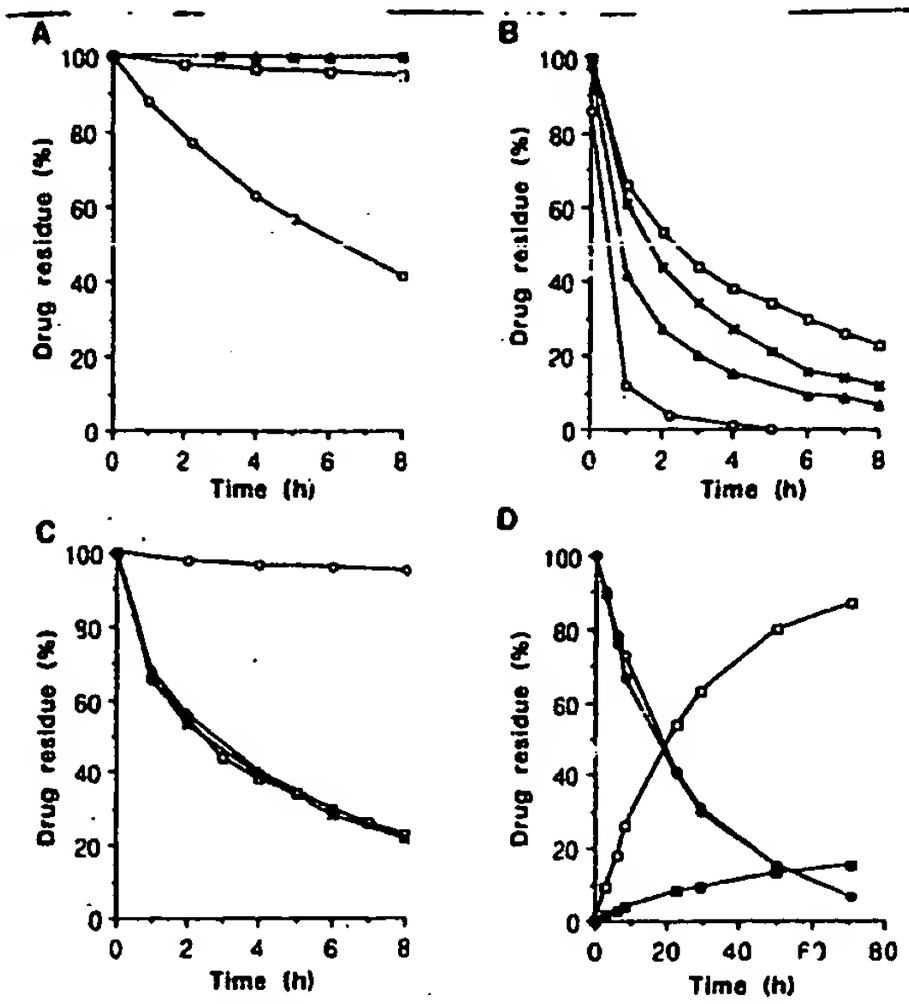


Figure 2. HPLC analysis of calf thymus DNA-treated and untreated drugs. Drugs (0.038 mM) were incubated with or without calf thymus DNA (1.1 mM bp) in 20% acetonitrile/10 mM phosphate buffer (pH 7.0) at 35 °C and the amounts of residual drugs or solvolysis products in the reaction mixtures were estimated by HPLC analysis at intervals (detector; Shimadzu SPD 10A; wavelength, 330 nm; column, unisil $_5C_{18}$-250A; elution buffer, 40–60% acetonitrile/50 mM phosphate buffer (pH 5.9); flow rate, 1 mL/min). (A) The rsidue of DUMA (○), DUMSA (×), 1 (□), and 2 (△) in the absence of DNA. (B) The residue of DUMA (○), DUMSA (×), 1 (□), and 2 (△) in the presence of DNA. (C) The residue of 1 in the absence of DNA (○) or in the presence of DNA. Reaction mixtures were subjected to HPLC directly (□), after DNA was precipitated by ethanol (△), or after extraction with 1-butanol (×). (D) Disappearance of KW-2189 (circle) and formation of 12 (square) in the absence (open symbol) or presence (closed symbol) of DNA.

2A shows the time course for the loss of drug residue. DUMA, containing the *altered*-CPI (cyclopropylpyrroloindole) alkylating subunit in the molecule, was subjected to solvolysis in 20% CH_3CN/0.01 M phosphate buffer (pH 7.0) at 35 °C to give the inactive hydrolyzed products 3 and 4 via nucleophilic attack of water at the C_4 and C_{4a} positions, respectively (Scheme 1). On the other hand, 1 afforded only one kind of hydrolyzed compound 7 very slowly (Figure 2A, Scheme 2), and DUMSA and 2 were so stable that no solvolysis products were observed under these conditions. Since under these aqueous neutral conditions no products were detected by HPLC except hydrolyzed compounds, the stability of DUMs might reflect the reactivity of the drug with a hydroxyl anion, that is, the electrophilicity of the cyclopropane of these drugs. The reactivity of the cyclopropane was significantly different among DUMs. Solvolysis of the *altered*-CPI unit of DUMA was faster than that of the CPI unit of other DUMs described in this study. C2 and C3 substituents on the CPI unit also influenced the rate of solvolysis. These results indicate that the electrophilicity of the cyclopropane depends on the A-ring structure of the left hand segment. Thus, the electrophilicity of the cyclopropane of these drugs is as follows: DUMA > 1 > DUMSA = 2. Recently, from the examination of HPLC analysis and biological activity of DUM–DNA adducts we have observed that the DNA alkylating reaction of DUMA, SA, and analogues is reversible.[12] The rate of regeneration of these drugs from the covalent DNA adducts (2 > DUMSA > 1 ≫ DUMA) was correlated to the electrophilicity of these drugs (described above). Independently, studies concerning the reversibility of DUMA and SA have been reported,[13] with results similar to ours.

Reactivity of DUMs with DNA. To investigate the reactivity of DUMs with DNA, we determined the amount of drug residue in the presence of calf thymus DNA (drug/DNA bp = 1/29) by reverse-phase HPLC analysis at intervals. In the absence of DNA the amount of drug decreased slowly and generated the hydrolyzed compounds as mentioned above. On the other hand in the presence of calf thymus DNA the rate of disappearance of drug was accelerated (Figure 2B), and no hydrolyzed products were detected. In the presence of DNA, only DUMA provided a small amount of hydrolyzed products 3 and 4. However, the yields were only 2.3 and 2.2%, respectively, after 5 h. In contrast, in the absence of DNA the yield of the hydrolyzed products was 41% (3:4 ≈ 1:1) after 5 h. These results suggest that the drugs bound to DNA covalently or noncovalently. The accelerated decrease of residual drugs in the presence of DNA was also observed when the supernatant was analyzed after DNA precipitation with ethanol, or when a 1-butanol extraction of the reaction mixture was analyzed by HPLC (Figure 2C). These precipitation or extraction procedures ensured that the noncovalently bound drug had been removed from the DNA. After the complete disappearance of the free drug followed by ethanol precipitation, we detected different CD spectra of DNA induced by these drugs (data not shown). Distamycin A, which is known as a strong minor groove binder without alkylating ability, did not exhibit the accelerated loss of residual drug in the presence of DNA when the reaction mixture was subjected to HPLC directly. These results imply that DUMs immediately alkylated DNA after the noncovalent binding stage or that DUMs which had bound to DNA noncovalently could be removed from DNA in the ODS HPLC column system and subsequently be chromatographed. Since no hydrolyzed products were observed in the presence of DNA, the amount of drug which disappeared in the presence of DNA is considered to be equal to the amount of drug converted to the covalent DNA adducts.[14] Though all of the DUMs described here (except for KW-2189) provided faster disappearance in the presence of DNA than in the absence of DNA, these drugs differed in the rate of disappearance in the presence of DNA (Figure 2B). The results imply that the rate of covalent reaction with DNA is affected by the A-ring structure of the left hand segment of DUM. The reactivity of the drugs with DNA did not strictly correlate to the electrophilicity of the drugs. DUMA, which was much more unstable than other DUMs in the absence of DNA (Figure 2A), showed higher reactivity with DNA than other drugs (Figure 2B). Thus the higher reactivity of DUMA with DNA might be due mainly to the strong electrophilicity of its cyclopropane subunit. However, the rate of alkylation of 2 was faster than that of other drugs except DUMA, though 2 was the most stable in the absence of DNA. From these results, two factors for DNA alkylation of DUMs could be inferred: (1) the electrophilicity of the cyclopropane subunit and (2) steric effects of the C_3 substituent of the left hand segment. One more factor namely, the effect of hydrophobic interaction of the right hand segment with the minor groove of DNA, was proposed for understanding the reaction between DNA and CC-1065 analogues.[15] However, all of the right hand segments of the DUMs described here are trimethoxy indole-types, suggesting that the effect of the right hand segment was

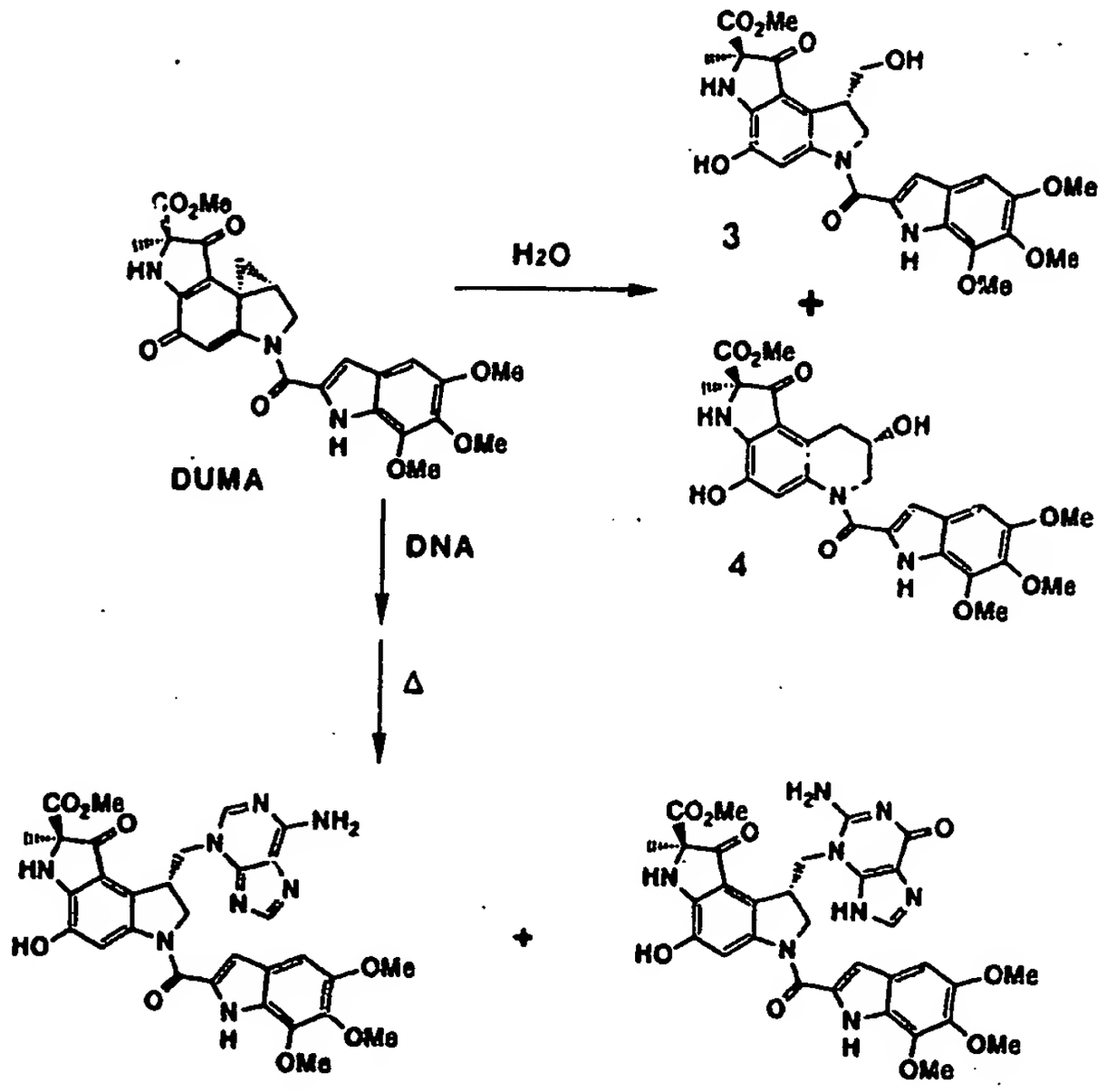
(12) Asai, A.; Nagamura, S.; Saito, H.; Takahashi, I.; Nakano, H. *Nucleic Acids Res.* 1994, *22*, 88.

(13) Boger, D. L.; Yun, W. *J. Am. Chem. Soc.* 1993, *115*, 9872.

(14) This thinking provided us with a useful method for investigation of the drug–DNA interaction and, furthermore, for designing new antitumor agents. In fact, we obtained a good correlation between the cytotoxicity of DUM derivatives in which the right hand segment was substituted and the amount of loss of these drugs at a specific time interval in the presence of DNA (unpublished observation).

(15) Boger, D. L.; Munk, S. A.; Zarrinmayeh, H. *J. Am. Chem. Soc.* 1991, *113*, 3980.

Scheme 1



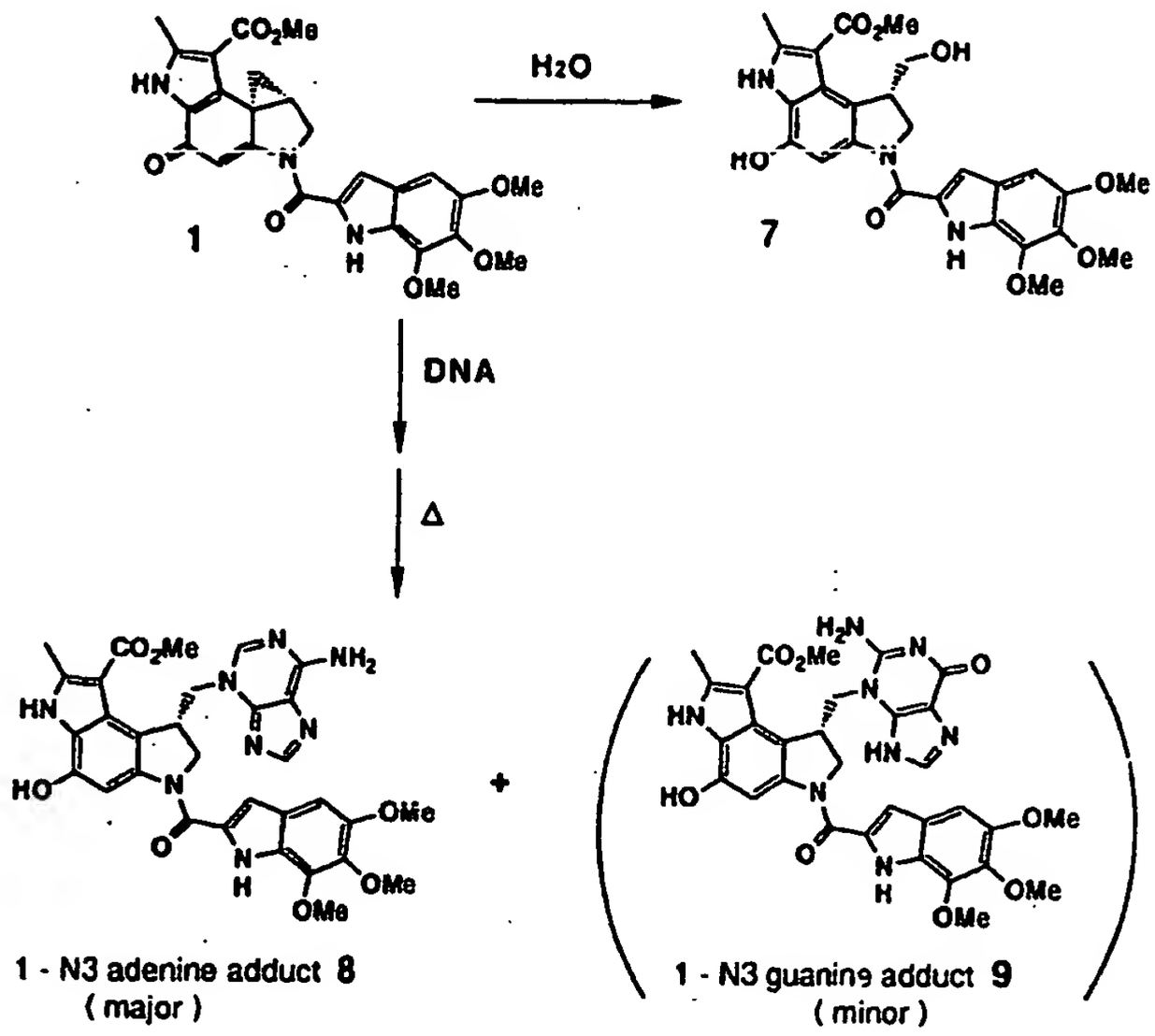
negligible in our examinations. Thus, noncovalent or covalent binding of **1** to DNA might be sterically hindered by the methyl ester substituent of C_3. Therefore, the alkylating reaction of **1** would be slower than **2**, although the reactivity of its cyclopropane would be higher than that of **2**. This assumption was supported by the weaker cytotoxicity of the C_3-isopropylester derivative of **1** (unpublished results).

Isolation and Characterization of DUM–Base Adducts. In order to gain further insight into the mechanisms of DNA binding by DUMs, we prepared and isolated DUM–base adducts and obtained both ^1H and ^{13}C NMR spectra. DUMA was incubate with calf thymus DNA (drug/DNA bp = 1/20) at 37 °C for 24 h. When the DUMA–DNA adduct was heated at 100 °C for 30 min, after removal of unbound DUMA, **3** and **4** by 1-butanol extraction and ethanol precipitation, two compounds with the DUMA chromophore were unexpectedly released from the DNA. Characterization of these compounds by ^1H and ^{13}C NMR, 2D ^1H–^1H NOESY, ^1H–^{13}C HSQC, and ^1H-^{13}C HMBC indicated that one was the DUMA–N3 adenine adduct **5**[8,11] which had been already fully characterized by Boger et al. The other was found to be the DUMA–N3 guanine adduct **6** (The yields of the adenine adduct and the guanine adduct were 56 and 28%, respectively.). The structure of **6** was initially identified by a methylation experiment.,[16] In the ^1H-NMR spectrum of **6**, all of the resonances were comparable to those of DUMC$_2$. One additional nonexchangeable resonance at 7.64 ppm (1H) and two exchangeable resonances at 7.07 (2H) and 13.00 ppm (1H) were found (Table 1). The only base residue that gives one nonexchangeable resonance is guanine. Furthermore, the chemical shift value of this resonance showed a close correlation with the guanine H_8 resonance. This data suggests that **6** is a DUMA–guanine adduct. This was supported by the secondary ion mass spectrum (SIMS) of **6** which gave the corresponding molecular ion peak $(M + H)^+$ at m/z 659. With respect to the ^{13}C resonance, five resonances were observed which were attributable to the guanine moiety in **6** (Table 2). One of them at 137.3 ppm was assigned as guanine C_8 by the HSQC experiment. Furthermore, in HMBC experiments, four cross peaks were observed connecting the guanine H_8 proton with the ^{13}C resonances of 147.3 and 110.8 ppm, and the C_{4a}–H proton with the ^{13}C resonances of 147.3 and 153.2 ppm (Figure 3 and Table 2). From these results all of the ^1H and ^{13}C resonances could be assigned and the C_{4a}–guanine N_3 bond was identified. In the HMBC experiments of **5** four cross peaks were also observed connecting the adenine H_8 proton with the resonance of ade-C_4 (150.3 ppm) and ade-C_5 (120.3 ppm) and the C_{4a}–H proton with the ^{13}C resonances of ade-C_2 (143.1 ppm) and ade-C_4 (150.3 ppm). The same treatment of **1** with calf thymus DNA also gave two adducts. These products were identified as **1**–N3 adenine adduct **8** and **1**–N3 guanine adduct **9**. (The yields of the adenine adduct and the guanine adduct were 74 and 7%, respectively.) Due to its low yield, **9** could not be fully characterized but its ^1H NMR spectra and SIMS indicated its structure as the N3–guanine adduct. In contrast, DUMSA and **2** provided N3 adenine adducts, **10** (yield; 81%) and **11** (yield; 79%), predominantly with trace amounts of minor products by thermal depurination reaction of these DNA adducts.

Selectivity of DUMs to Adenine. The DNA alkylating reaction of DUMA followed by thermal depurination provided DUMA–N3 adenine and DUMA–N3 guanine adducts (adenine/guanine = 2:1) when high concentrations of the drug were used (drug/DNA bp = 1/20, see Experimental Section). The ratio of the two adducts was correlated to the ratio of drug/DNA base pair (bp), that is, low values of drug/DNA bp resulted in selective

(16) Sugiyama, H.; Kazushige, O.; Chan, K. L.; Hosoda, M.; Asai, A.; Saito, H.; Saito, I. *Tetrahedron Lett.* **1993**, *34*, 2179.

Scheme 2



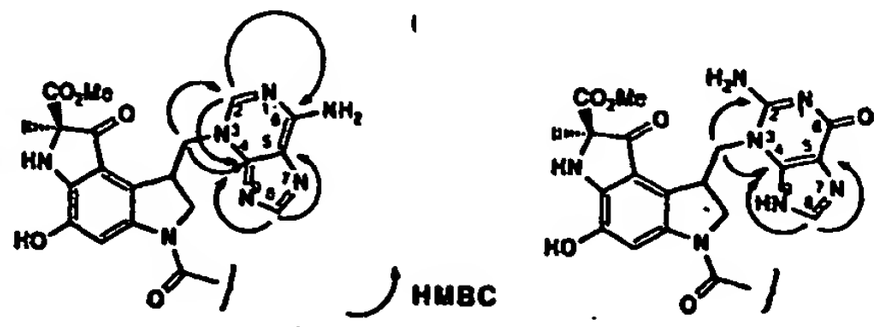


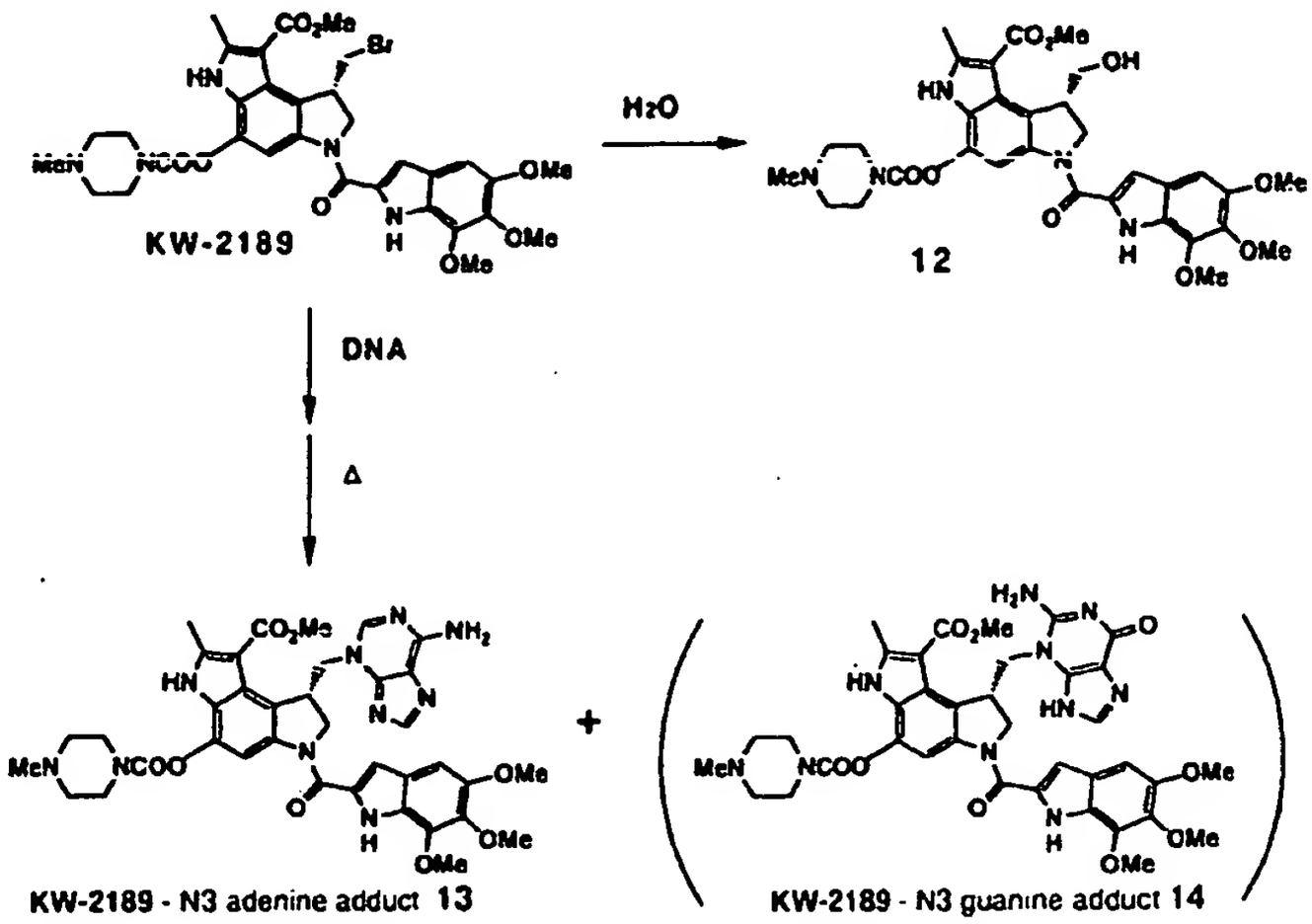
Figure 3. Correlation of ^{1}H–^{13}C long-range couplings observed in HMBC spectrums of 5 and 6.

alkylation of adenine (Table 3). These results suggest the DUMA alkylates N3–adenine selectively at first and that N3–guanine is the second target for DUMA. On the other hand, 1 afforded both its adenine and guanine adduct, with higher selectivity to adenine (adenine/guanine = 10:1) than that of DUMA. DUMSA and 2 gave only their adenine adducts. Since these reactions were performed under the same conditions (see Experimental Section), the alkylating selectivity to N3 adenine is 2 = DUMSA > 1 > DUMA. Interestingly, a correlation between the selectivity to adenine and the electrophilicity of the cyclopropane subunit was observed. DUMA, containing the most reactive cyclopropane alkylated both adenine and guanine. The most stable analogues 2 and DUMSA, containing less reactive cyclopropanes, demonstrated the greatest specificity for adenine. Compound 1, which contains a cyclopropane less reactive than that of DUMA but more reactive than that of DUMSA and 2 (Figure 2A), alkylated adenine more selectively than did DUMA. It was presumed that DUMA possessed lower sequence selectivity due to the higher reactivity of its cyclopropane. The consensus sequences to which DUMA and CC-1065 bind through N3 adenine have been identified.[9,10] Our results, along with already published reports,[16,17] reveal that DUMA and 1 bind to other sequences, mediating N3 guanine adduct formation. Boger et al. reported that hydrophobic interactions of the right hand segment with the minor groove of DNA affect the sequence selectivity of DUMA, CC-1065, and their analogues.[15] Now we have showed the effect of the electrophilicity of the cyclopropane, which was dependent on the A-ring structure of the left hand segment, on the selectivity for adenine vs guanine. This effect might also extend to the sequence selectivity of these drugs.

A Novel Property of KW-2189 for DNA Alkylation. KW-2189, the synthetic derivative of 1 which possesses improved *in*

(17) Mitchell, M. A.; Weiland, K. L.; Aristoff, P. A.; Johnson, P. D.; Dooley, T. P. *Chem. Res. Toxicol.* **1993**, *6*, 421.

Scheme 3



vivo antitumor activity, was designed as a prodrug requiring enzymatic hydrolysis of the carbamoyl moiety, followed by regeneration of **1**. As mentioned above **1** alkylates DNA at the N3 adenien more selectively than does DUMA. Surprisingly, we discovered that KW-2189 itself alkylated DNA without release of the carbamoyl moiety. Incubation of KW-2189 in 20% CH_3CN/phosphate buffer (pH 7.0) at 35 °C resulted in the formation of the inactive hydroxyl compound **12** quantitatively. No carbamoyl-hydrolyzed products, such as **1**, were detected by HPLC. However, in the presence of DNA the formation of **12** was not quantitative though the rate of disappearance of KW-2189 was same as in the absence of DNA (Figure 2D). Furthermore, we obtained the KW-2189–N3 adenine adduct **13** and the guanine adduct **14** (4:1) from calf thymus DNA treated with KW-2189 (drug/DNA bp = 1:20) by a thermally induced depurination reaction. Due to its low yield, **14** could not be fully characterized, but its ^1H NMR spectra and SIMS indicated the structure of the N3–guanine adduct. Interestingly, we also found that **17** was hydrolyzed to give **12** in 20% CH_3CN/phosphate buffer (pH 7.0) at 35 °C. Incubation of **17** with DNA, followed by thermal depurination, afforded the same adducts **13** and **14** (Schemes 3). In contrast, DNA alkylation by the carbamoyl derivatives of $DUMB_2$ and B_1, **15** and **16**, which were more stable than KW-2189 in aqueous solution, was not detected under the conditions described above.

For the mechanism of DNA alkylation by KW-2189 and **17** we propose the rate-determining formation of the reactive cyclopropane **18** (Figure 4) in aqueous solution. No intermediates could be detected by HPLC analysis under the conditions in which KW-2189 was converted to **12**. It would be difficult to detect the intermediate **18** due to its lability. It is noteworthy that **17**, containing the fused six-membered ring, also afforded the five-membered ring product **12** and adducts **13** and **14** with an intact carbamoyl moiety. Carbamoyl derivatives of the DUMA-type, containing an *altered*-CPI alkylating subunit, could not generate a reactive species like **18**. These agents were rather stable in aqueous condition. Although it is not concluded that DNA alkylation by KW-2189 itself, without enzymatic activation, is responsible for its high efficacy *in vivo*, further evaluation of KW-2189 is underway. Its antitumor activity and its DNA alkylation in cells will be reported in the near future.

Experimental Section

Instrumentation and Reagents. DUMA and DUMSA were isolated.[16] **1**,[18a] **2**,[18a] **15**,[18b] and KW-2189[18a] were synthesized from $DUMB_2$, and **16**[18c] and **17** were synthesized for $DUMB_1$ in our laboratories. The

(18) Saito, H. et. al. Eur. Pat. Appl. (a) EP 406749; *Chem. Abstr.* **1991**, *115*, 8427h. (b) EP 499130; *Chem. Abstr.* **1992**, *118*, 38908v. (c) EP 468400; *Chem. Abstr.* **1992**, *116*, 194292d. The papers about synthetic details and biological activity of these compounds are in preparation.

Table 1. ^{1}H NMR of 5 and Related Compounds (DMSO-d_6, 500 MHz)

	5[a]	8	11[a]	10	6	DUMC$_2$
			Left Hand Segment			
C8-OH (1H, br)	10.15	9.56	9.65	9.82	10.20	10.20
C7-H (1H, brs)	8.03	7.68	7.56	7.77	8.06	8.03
N1-H (1H, brs)	7.23	11.96	10.83	11.60	7.42	7.03
C4a-H (1H)	4.64 (dd J = 5.4, 13.7 Hz)	4.52 (m)	4.69 (dd J = 4.8, 13.5 Hz)	4.65 (dd J = 5.0, 13.4 Hz)	4.32 (dd J = 9.6, 14.7 Hz)	4.03 (dd J = 3.0, 10.0 Hz)
C4a-H (1H)	4.49 (dd J = 7.1, 13.7 Hz)	4.36 (dd J = 7.8, 13.2 Hz)	4.40 (dd J = 8.3, 13.5 Hz)	4.48 (dd J = 8.4, 13.4 Hz)	4.00 (dd J = 3.7, 14.7 Hz)	3.86 (dd J = 6.9, 10.0 Hz)
C5-H (1H)	4.68 (dd J = 2.7, 10.9 Hz)	4.75 (d J = 10.5 Hz)	4.44 (m)	4.51 (m)	4.64 (d J = 9.9 Hz)	4.62 (dd J = 11.0, 11.0 Hz)
C5-H (1H)	4.41 (dd J = 10.9, 10.9 Hz)	4.24 (dd J = 10.5, 10.5 Hz)	4.44 (m)	4.51 (m)	4.37 (dd J = 9.9, 9.9 Hz)	4.32 (dd J = 4.0, 11.0 Hz)
C4-H (1H, m)	4.22	4.58	4.24	4.37	4.07	3.99
C2-CO_2CH_3 (3H, s)	3.58	3.83 (C3-CO_2Me)		3.88	3.63	3.61
C2-CH_3 (3H, s)	1.41	2.65	2.34		1.42	1.47
C3-H (1H, s)			5.88	6.91		
			Right Hand Segment			
N1'-H (1H, brs)	11.19	11.04	11.16	11.21	11.29	11.34
C4'-H (1H, s)	6.90	6.84	6.89	6.89	6.87	6.95
C3'-H (1H, d)	6.83 (J = 2.0 Hz)	6.72 (J = 2.0 Hz)	6.83 (J = 1.7 Hz)	6.87 (J = 2.0 Hz)	6.84 (J = 2.1 Hz)	6.87 (J = 2.0 Hz)
C5'-CH_3 (3H, s)	3.92	3.94	3.93	3.93	3.93	3.92
C6'-CH_3 (3H, s)	3.81	3.80	3.80	3.81	3.79	3.81
C7'-CH_3 (3H, s)	3.79	3.79	3.79	3.79	3.78	3.79
			Base			
ade-C2-H (1H, s)	7.93	7.79	8.17	8.14	13.00 (gua-N9-H, 1H, br)	
ade-NH_2 (2H, br)	7.85	7.73	8.10	7.88	7.64 (gua-C8-H, 1H, s)	
ade-C8-H (1H, s)	7.64	7.49	8.04	7.81	7.07 (gua-NH_2, 2H, s)	

[a] Operated at 400 MHz.

preparation and characterization of 3, 4, 7, and 12, and full characterization of 1 and 2 are provided in the supplementary material. Calf thymus DNA was purchased from Sigma. All of the NMR experiments were performed on Bruker AM500 or AM400 spectrometers operating at 500 or 400 MHz for proton and 125 or 100 MHz for carbon observations. Fast atom bombardment mass spectra (FABMS) and fast atom bombardment high-resolution mass spectra (FABHRMS) were run on a HX110A, secondary ion mass spectra (SIMS) and electron impact mass spectra (EIMS) were run on a Hitachi M80B spectrometer.

General Procedure for DNA-Alkylation by DUMs and Isolation of DUM–Base Adducts. A solution of drug (7.1×10^{-3} mmol) in *N,N*-dimethylformamide (1 mL) was treated with calf thymus DNA (90 mg, 0.14 mmol bp) in 10 mM potassium phosphate buffer (pH 7.0, 9 mL), and mixture was incubated at 37 °C for 24 h (7 days for KW-2189). One mL of 3 M aqueous potassium acetate was added, and, the DNA was precipitated with 25 mL of ethanol. The precipitated DNA was dissolved in 10 mM potassium phosphate buffer (pH 7.0, 10 mL) and 1-butanol (10 mL), and the solution was heated at 100 °C for 15 min with stirring. After cooling to room temperature, the 1-butanol layer was separated. The aqueous layer was heated with 1-butanol (10 mL) for 15 min at 100 °C. The combined 1-butanol layers were concentrated. After the residue was dissolved in 50% aqueous methanol, the sample was subjected to HPLC (YMC R335-20, 50 × 500 mm) with 40–50% acetonitrile/50 mM potassium phosphate buffer (pH 5.9), solvent at a flow rate of 20 mL/min. The eluted fraction that contained the DUM chromophore (detector; Shimadzu CR4A) was collected and concentrated. The samples were desalted on a Dianion HP20 column, concentrated, and dried in vacuo to yield DUM–base adducts. By following this procedure DUMA afforded both DUMA–N3 adenine adduct 5 (2.5 mg, 55%) and DUMA–N3 guanine adduct 6 (1.3 mg, 28%) as yellow solids. 5: ^{1}H NMR (DMSO-d_6, 400 MHz) Table 1, ^{13}C NMR (DMSO-d_6, 100 MHz) Table 2; SIMS, m/e 643 (M^+ + H); FABHRMS, m/e 643.2267 ($C_{31}H_{30}N_8O_9$ + H^+ requires 643.2265). 6: ^{1}H NMR (DMSO-d_6, 500 MHz) Table 1, ^{13}C NMR (DMSO-d_6, 125 MHz) Table 2; SIMS, m/e 659 (M^+ + H); FABHRMS, m/e 659.2221 ($C_{31}H_{30}N_8O_{10}$ + H^+ requires 659.2214).

By following the procedure described above 1 afforded both the 1-N3 adenine adduct 8 (3.3 mg, 74%) and the 1–N3 gaunine adduct 9 (0.4 mg, 7%) as pale yellow solids. 8: ^{1}H NMR (DMSO-d_6, 500 MHz) Table 1, ^{13}C NMR (DMSO-d_6, 125 MHz) Table 2; SIMS, m/e 627 (M^+ + H); FABHRMS, m/e 627.2332 ($C_{31}H_{30}N_8O_7$ + H^+ requires 627.2315). 9: ^{1}H NMR (DMSO-d_6, 500 MHz) ppm; 12.70 (1H br gua-C9-H), 12.00 (1H, brs, N1-H), 11.29 (1H, brs, N1'-H), 10.15 (1H, br, C8-OH), 7.68 (1H, s, C7-H), 7.31 (1H, s, gua-C8-H), 7.06 (2H, br, gua-C2-NH_2), 6.74 (1H, s, C4'-H), 6.57 (1H, d, J = 2.1 Hz C3'-H), 4.43 (1H, m, C4-H), 4.22 (1H, dd, J = 7.0, 10.9 Hz, C5-H), 4.15 (1H, dd, J = 11.9, 14.5 Hz, C4a-H), 4.05 (1H, d, J = 10.9 Hz, C5-H), 3.95 (3H s C5'-CH_3), 3.93 (1H, dd, J = 4.5, 14.5 Hz, C4a-H) 3.85 (3H, s, C3-CO_2CH_3), 3.80 (3H, s, C6'-CH_3), 3.76 (3H, s, C7'-CH_3), 2.64 (3H, s, C2-CH_3); SIMS, m/e 643 (M^+ + H); FABHRMS, m/e 643.2268 ($C_{31}H_{30}N_8O_8$ + H^+ requires 643.2265).

By following the procedure described above DUMSA afforded the DUMSA–N3 adenine adduct 10 (3.5 mg, 81%) as a pale yellow solid. 10: ^{1}H NMR (DMSO-d_6, 400 MHz) Table 1, ^{13}C NMR (DMSO-d_6, 100 MHz) Table 2; FABMS, m/e 613 (M^+ + H); FABHRMS, m/e 613.2167 ($C_{30}H_{29}N_8O_7$ + H^+ requires 613.2159).

By following the procedure described above 2 afforded the 2–N3 adenine adduct 11 (3.2 mg, 79%) as a pale yellow solid. 11: ^{1}H NMR (DMSO-d_6, 500 MHz) Table 1, ^{13}C NMR (DMSO-d_6, 125 MHz) Table 2; SIMS, m/e 569 (M^+ + H); FABHRMS, m/e 569.2268 ($C_{29}H_{29}N_8O_5$ + H^+ requires 569.2261).

By following the procedure described above KW-2189 afforded both the KW-2189-N3 adenine adduct 13 (3.1 mg, 58%) and the KW-2189–N3 guanine adduct 14 (0.8 mg, 15%) as pale yellow solids. 13: ^{1}H NMR (DMSO-d_6, 500 MHz) ppm; 12.01 (1H, br, N1-H), 11.10 (1H, brs, N1'-H), 7.93 (1H, s, ade-C2-H), 7.85 (1H, brs, C7-H), 7.78 (2H, br, ade-C6-NH_2), 7.50 (1H, s, ade-C8-H), 6.85 (1H, s, C4'-H), 6.79 (1H, d, J = 2.1 Hz, C3'-H), 4.82 (1H, d, J = 10.8 Hz, C5-H), 4.71 (1H, m, C4-H), 4.56 (1H, dd, J = 3.3, 13.3 Hz, C4a-H), 4.43 (1H, dd, J = 8.3, 13.3 Hz, C4a-H), 4.32 (1H, dd, J = 8.4, 10.8 Hz, C5-H), 3.94 (3H, s, C5'-CH_3), 3.85 (3H, s, C3-CO_2CH_3), 3.80 (3H, s, C6'-CH_3), 3.79 (3H, s, C7'-CH_3), 3.71 (2H, br, C2''-H_2 or C6''-H_2), 3.50 (2H, br, C2''-H_2 or C6''-H_2), 2.70 (3H, s, C2-CH_3), 2.40 (4H, br, C3''-H_2 and C5''-H_2), 2.26 (3H, s, N1''-CH_3); ^{13}C NMR (DMSO-d_6, 125 MHz) ppm; 165.2 (C2-CO_2CH_3), 160.2 (C2a'), 154.5 (ade-C6), 152.5 (C4a''), 152.1 (ade-

Table 2. ^{13}C NMR of 5 and Related Compounds (DMSO-d_6, 125 MHz)

	5[a]	8	11[a]	10	6	DUMC$_2$
			Left Hand Segment			
C-3	197.6	103.2	96.5	105.8	199.0	196.6
CO_2CH_3	169.1	165.6		161.3	169.0	169.6
C-8	149.0	142.9	142.2	143.3	149.1	150.1
C-8a	143.5	122.6	123.3	125.7	143.7	144.2
C-6a	136.2	138.9	136.4	137.9	136.3	137.7
C-3a	118.0	124.8	125.7	124.1	117.9	119.5
C-3b	114.3	111.9	110.7	112.7	114.0	115.6
C-7	110.8	98.1	98.1	100.3	111.4	112.5
C-2	69.8	145.0	136.2	127.9	70.2	71.2
C-5	54.4	54.1	54.0	54.1	54.2	55.0
CO_2CH_3	52.5	50.6		51.7	52.8	53.4
C-4a	52.1	52.2	52.3	52.3	46.7	46.4
C-4	39.4	42.2	38.9	39.0	37.9	42.3
2-CH3	20.0	14.6	13.2		19.9	22.0
			Right Hand Segment			
C-2′a	159.3	160.0	159.2	159.6	158.7	160.5
C-5′	149.0	150.5	148.9	149.9	150.9	150.4
C-6′	139.6	139.4	139.4	139.6	139.7	140.9
C-7′	138.9	138.9	139.0	138.4	139.0	138.7
C-2′	130.9	131.6	131.7	131.4	130.0	129.1
C-7′a	125.0	124.8	124.8	125.0	125.1	126.0
C-3′a	123.0	123.1	123.3	123.1	123.1	123.5
C-3′	105.6	105.2	105.0	105.4	105.6	107.9
C-4′	97.9	98.0	98.0	98.0	97.9	98.0
C6′-OCH_3	61.0	61.0	61.0	61.0	61.0	61.5
C7′-OCH_3	60.8	60.9	60.8	60.9	60.9	61.2
C5′-OCH_3	55.9	56.0	54.0	56.0	55.9	56.4
			Base			
ade-C2	143.1	143.2	143.1	143.6	153.2 (gua-C2)	
ade-C4	150.3	150.5	149.9	149.9	147.3 (gua-C4)	
ade-C5	120.3	120.1	120.5	120.5	110.8 (gua-C5)	
ade-C6	154.6	154.5	154.9	155.0	161.5 (gua-C6)	
ade-C8	152.1	152.0	152.5	152.5	137.3 (gua-C8)	

[a] Operated at 100 MHz.

Table 3. Effect of Drug/DNA bp Ratio on the Selective Adenine Alkylation[a]

drug/DNA bp	adenine adduct/ guanine adduct	drug/DNA bp	adenine adduct/ guanine adduct
0.1	1.4:1	0.0125	10:1
0.05	2.1:1	0.00625	11:1
0.025	5.1:1		

[a] Various concentrations of DUMA were incubated with calf thymus DNA (1.5 mM bp) in 10% *N,N*-dimethylformamide/10 mM potassium phosphate buffer at 35 °C for 8 h. After 1-butanol extraction and ethanol precipitation, these DNA samples were dissolved in 10 mM potassium phosphate buffer and heated at 100 °C for 10 min. Reaction mixtures were subjected to HPLC analysis (detector, Shimadzu SPD 10A; wavelength, 330 nm; column, unisil $_5C_{18}$-250A; elution buffer, 40% acetonitrile/50 mM phosphate buffer, pH 5.9; flow rate, 1 mL/min). These alkylating and depurination conditions afforded 80–85% yields of DUM–base adducts.

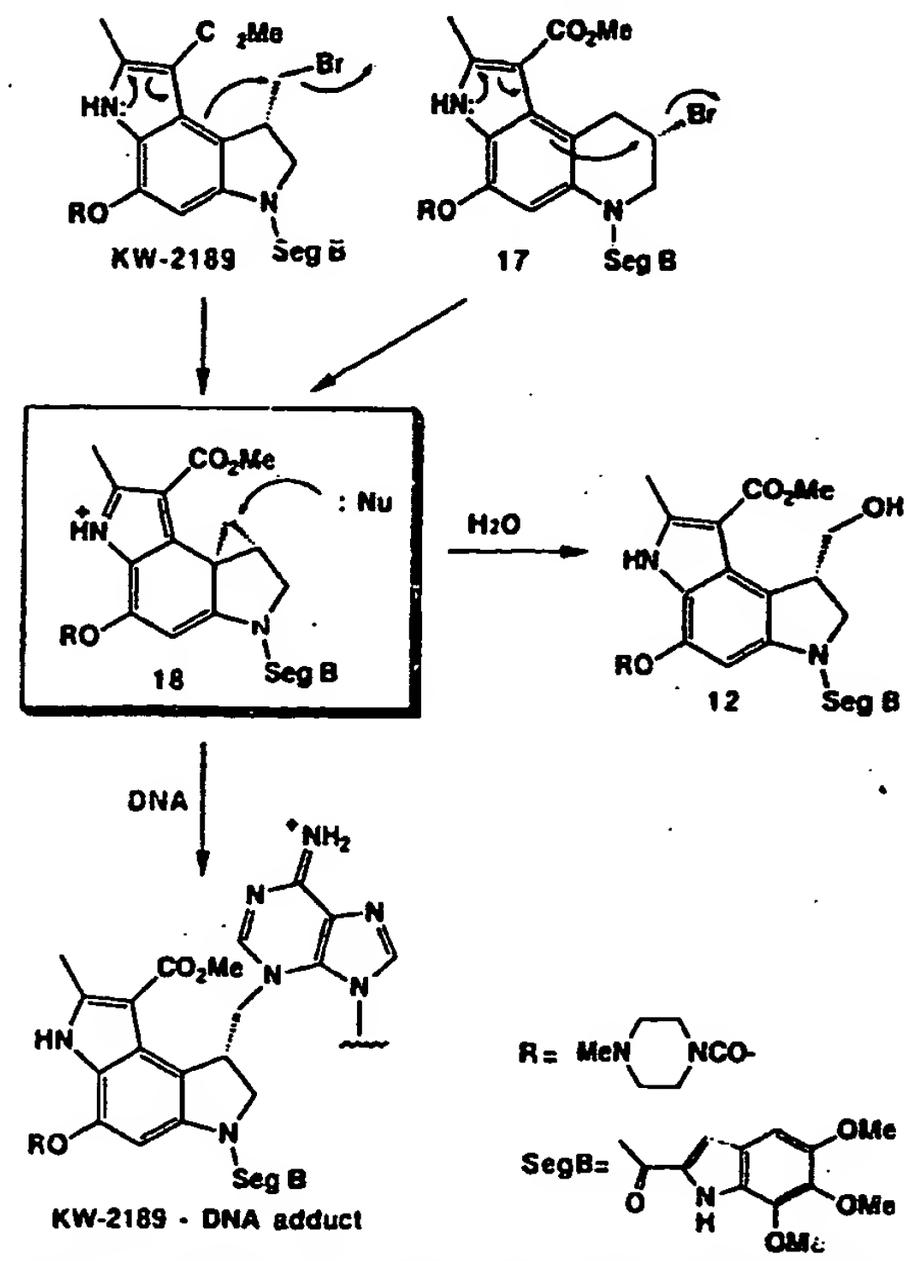


Figure 4. Proposed mode of action of KW-2189 for DNA alkylation.

C8), 150.5 (ade-C4), 148.9 (C5′), 146.4 (C2), 143.2 (ade-C2), 139.5 (C6′), 138.9 (C7′), 138.2 (C6a), 135.9 (C8), 131.2 (C2′), 125.7 (C8a), 124.9 (C7a′), 124.4 (C3a), 123.0 (C3a′), 120.2 (ade-C5), 118.5 (C3b), 105.8 (C7), 105.4 (C3′), 103.4 (C3), 99.9 (C4′), 61.0 (C6′-CH_3), 60.9 (C7′-CH_3), 55.9 (C5′-CH_3), 54.1 (C2″ or C6″), 53.9 (C2″ or C6″), 53.5 (C5), 51.9 (C4a), 50.8 (C3-CO_2CH_3), 45.7 (N1″-CH_3), 44.1 (C3″ or C5″), 43.2 (C3″ or C5″), 42.5 (C4), 14.6 (C2-CH_3); SIMS, *m/e* 753 (M^+ + H); FABHRMS, *m/e* 753.3129 ($C_{37}H_{40}N_{10}O_8$ + H^+ requires 753.3109). **14**: ^1H NMR (DMSO-d_6, 500 MHz) ppm; 12.70 (1H, br, gua-C9-H), 12.04 (1H, brs, N1-H), 11.32 (1H, brs, N1′-H), 7.83 (1H, s, C7-H), 7.32 (1H, s, gua-C8-H), 7.07 (2H, br, gua-C2-NH_2), 6.76 (1H, s, C4′-H), 6.60 (1H, d, *J* = 2.0 Hz, C3′-H), 4.56 (1H, m, C4-H), 4.29 (1H, dd, *J* = 7.2, 10.9 Hz, C5-H), 4.19 (1H, dd, *J* = 11.8, 14.7 Hz, C4a-H), 4.10 (1H, d, *J* = 10.9 Hz, C5-H), 4.00 (1H, dd, *J* = 4.4, 14.7 Hz, C4a-H), 3.95 (3H, s, C5′-CH_3), 3.89 (3H, s, C3-CO_2CH_3), 3.80 (3H, s, C6′-CH_3), 3.76 (3H, s, C7′-CH_3), 3.72 (2H, br, C2″-H_2), 3.49 (2H, br, C6″-H_2), 2.70 (3H, s, C2-CH_2), 2.46 (4H, br, C3″-H_2 and C5″-H_2), 2.26 (3H, s, N1″-CH_3); SIMS, *m/e* (rel intensity) 769 (M^+ + H, 24), 234 (base); FABHRMS, *m/e* 769.3071 ($C_{37}H_{40}N_{10}O_9$ + H^+ requires 769.3058).

Acknowledgment. We thank Dr. Mayumi Yoshida and Mr. Singo Kakita for measuring NMR spectra and for providing helpful discussions.

Supplementary Material Available: Full details of the preparation and characterization of compounds **3**, **4**, **7**, and **12** and the spectral and analytical data for compounds **1** and **2** (4 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

ASAI
4171-4177

Instrumentation and reagents.

All of the NMR experiments were performed on Bruker AM500 or AM400 spectrometers operating at 500 or 400MHz for proton and 125 or 100MHz for carbon observations. Fast atom bombardment mass spectra (FABMS) and fast atom bombardment high resolution mass spectra (FABHRMS) were run on a HX110A, secondary ion mass spectra (SIMS) and electron impact mass spectra (EIMS) were run on a Hitachi M80B spectrometer. Infrared spectra (IR) were recorded on a JASCO IR-810. For column chromatography, silica gel (SiO_2, Wako C-200) was used. Analytical thin-layer chromatography (TLC) was performed on silicagel 60 F254 plate (Merck).

compound 1 [18a]

^{1}H-NMR ($CDCl_3$, 400MHz) ppm; 11.35 (1H brs), 9.35 (1H brs), 7.10 (1H s), 6.95 (1H d J=2.2Hz), 6.81 (1H s), 4.45 (2H m), 4.08 (3H s), 3.94 (3H s), 3.90 (3H s), 3.82 (3H s), 3.66 (1H m), 2.63 (3H s), 2.38 (1H dd J=3.7, 7.5Hz), 1.36 (1H dd J=3.7, 4.9Hz). ^{13}C-NMR ($CDCl_3$, 100MHz) ppm; 177.4, 165.1, 162.0, 161.2, 150.4, 142.7, 141.0, 138.9, 132.1, 128.8, 128.7, 126.2, 123.4, 111.4, 108.2, 107.5, 97.7, 61.5, 61.2, 56.3, 54.8, 50.9, 32.3, 24.3, 24.2, 14.8. IR (KBr) cm^{-1}; 3470, 3225, 2934, 1700, 1637, 1607, 1385, 1295, 1264, 1106. FABMS, m/e 492 ($M^+ + H$); FABHRMS, m/e 492.1761 ($C_{26}H_{25}N_3O_7 + H^+$ requires 492.1771)

compound 2 [18a]

^{1}H-NMR ($CDCl_3$, 500MHz) ppm; 10.02 (1H brs), 9.29 (1H brs), 6.94 (1H d J=2.3Hz), 6.91 (1H s), 6.77 (1H s), 5.67 (1H m), 4.37 (2H m), 4.07 (3H s), 3.93 (3H s), 3.89 (3H s), 2.69 (1H m), 2.37 (3H s), 1.70 (1H dd J=4.6, 7.4Hz), 1.53 (1H dd J=4.6, 4.6Hz). ^{13}C-NMR ($CDCl_3$, 125MHz) ppm; 176.8, 161.1, 159.4, 150.4, 141.0, 138.9, 136.5, 132.2, 128.8, 128.3, 126.2,

J. Am. Chem. Soc. v 116
o. 4171 Asai

123.3, 112.6, 107.5, 101.2, 97.8, 61.5, 61.2, 56.3, 54.8, 31.6, 25.9, 23.6, 13.4. IR (KBr) cm^{-1}; 3455, 3295, 2938, 1636, 1477, 1388, 1305, 1265. FABMS, m/e 434 ($M^+ + H$); FABHRMS, m/e 434.1715 ($C_{24}H_{23}N_3O_5 + H^+$ requires 492.1771)

compound 3 and 4

70% perchloric acid (0.05ml) was added to a solution of DUMA (50mg, 0.099mmol) in 74% acetonitrile / distilled water (4ml) and the mixture was stirred for 1h. 0.5M citric acid buffer (pH4) was added and the mixture was extracted with ethylacetate twice. The combined organic layers were washed with brine and dried (Na_2SO_4) and evaporated in vacuo, and the residue was purified by column chromatography. (SiO_2, 5% methanol in chloroform) to give diols 3 (24.8mg, 48%) and 4 (25.5mg, 49%) as yellow solids.

3: ^{1}H-NMR ($CD_3OD/CDCl_3$, 400MHz) ppm; 8.05 (1H brs), 7.03 (1H s), 6.91 (1H s), 4.60 (1H dd J=10.7, 3.7Hz), 4.54 (1H dd J=10.7, 9.0Hz), 4.09 (3H s), 4.00 (1H dd J=10.6, 4.1Hz), 3.94 (3H s), 3.91 (3H s), 3.85 (1H m), 3.75 (3H s), 3.58 (1H dd J=10.6, 7.9Hz) 1.63 (3H s). ^{13}C-NMR ($CD_3OD/CDCl_3$, 100MHz) ppm; 198.9, 170.4, 161.1, 151.3, 150.4, 144.3, 140.8, 139.5, 138.1, 130.8, 126.2, 124.5, 120.9, 115.9, 112.7, 107.5, 98.6, 71.5, 64.2, 61.8, 61.4, 56.6, 55.2, 53.5, 43.5, 21.4. FABMS, m/e 526 ($M^+ + H$); FABHRMS, m/e 526.1807 ($C_{26}H_{27}N_3O_9 + H^+$ requires 526.1825)

4: ^{1}H-NMR (CD_3OD, 400MHz) ppm; 6.84 (1H s), 6.76 (1H brs), 6.61 (1H s), 4.22 (1H m), 4.03 (1H dd J=12.6, 5.7Hz), 3.97 (3H s), 3.91 (1H dd J=12.6, 2.8Hz), 3.84 (3H s), 3.82 (3H s), 3.69 (3H s), 3.35 (1H dd J=18.3, 5.9Hz), 3.02 (1H dd J=18.3, 4.7Hz), 1.56 (3H s). ^{13}C-NMR (CD_3OD, 100MHz) ppm; 198.8, 170.4, 164.7, 152.8, 150.1, 142.1, 140.5, 139.5, 130.8, 130.5, 126.3, 123.9, 119.4, 118.6, 116.9, 108.5, 98.5, 71.5, 65.1,

J. Am. Chem. Soc. v 116
p. 4171 Asai

61.8, 61.4, 56.5, 53.4, 52.2, 32.4, 21.5. IR (KBr) cm^{-1}; 3300, 1735, 1684, 1622, 1520, 1424, 1399, 1306, 1247, 1105, 1048. FABMS, m/e 526 (M^+ + H); FABHRMS, m/e 526.1807 ($C_{26}H_{27}N_3O_9 + H^+$ requires 526.1825)

compound 7

70% perchloric acid (0.02ml) was added to a solution of 1 (10mg, 0.020mmol) in 74% acetonitrile / distilled water (1ml) and the mixture was stirred for 2h. 0.5M citric acid buffer (pH4) was added and the mixture was extracted with ethylacetate twice. The combined organic layers were washed with brine and dried (Na_2SO_4) and evaporated in vacuo, and the residue was purified by column chromatography (SiO_2, 5% methanol in chloroform to give diols 7 (8.2mg, 81%) as a pale yellow solid. ^1H-NMR (DMSO-d_6 400MHz) ppm; 11.72 (1H brs), 11.24 (1H brs), 9.90 (1H brs), 7.72 (1H brs), 6.97 (1H d J=1.9Hz), 6.94 (1H s), 4.72 (1H dd J=5.1, 5.1Hz), 4,48 (1H d J=9.5Hz), 4.40 (1H dd J=9.5, 9.5Hz), 4.01 (1H m), 3.94 (3H s), 3.82 (3H s), 3.79 (3H s), 3.78 (3H s), 3.58 (1H dd J=5.1, 5.1, 9.2Hz), 3.00 (1H dd J=5.1, 9.2, 9.2Hz), 2.60 (3H s). ^{13}C-NMR (DMSO-d_6, 100MHz) ppm: 165.0, 159.6, 149.0, 144.6, 142.1, 139.4, 138.9, 138.4, 131.8, 124.8, 123.5, 123.1, 122.4, 113.5, 105.3, 103.0, 98.2, 97.9, 63.4, 61.0, 60.8, 55.9, 53.9, 50.2, 43.5, 14.1. IR (KBr) cm^{-1}; 1595, 1490, 1442, 1320, 1223, 1161. FABMS, m/e 510 (M^+ + H); FABHRMS, m/e 510.1868 ($C_{26}H_{27}N_3O_8 + H^+$ requires 510.1876)

compound 12

A solution of hydochloric acid salt of KW-2189 (557mg, 0.67mmol) in 0.1M citric acid buffer (pH2.1, 100ml) was stirred at 60 °C. After four days the reaction mixture was poured into saturated sodium bicarbonate solution (50ml) and extracted with chloroform twice. The combined organic

layers were washed with brine and dried (Na_2SO_4) and evaporated in vacuo, and the residue was purified by column chromatography (SiO_2, 5% methanol in chloroform to give **12** (456mg, 95%) as a pale yellow solid.
^{1}H-NMR (DMSO-d_6 400MHz) ppm; 11.81 (1H brs), 11.27 (1H brs), 7.88 (1H brs), 7.01 (1H d J=1.9Hz), 6.95 (1H s), 4.84 (1H dd J=5.1, 5.1Hz), 4.54 (1H dd J=10.6, 1.7Hz), 4.49 (1H dd J=10.6, 8.0Hz), 4.14 (1H m), 3.94 (3H s), 3.82 (3H s), 3.81 (3H s), 3.80 (3H s), 3.69 (2H br), 3.61 (1H ddd J=5.1, 5.1, 9.7Hz), 3.47 (2H br), 3.10 (1H ddd J=5.1, 9.7, 9.7Hz), 2.67 (3H s), 2.41 (4H br), 2.26 (3H s). ^{13}C-NMR (DMSO-d_6, 100MHz) ppm; 164.8, 159.9, 152.6, 149.1, 146.1, 139.6, 139.0, 138.0, 135.2, 131.5, 125.6, 125.0, 124.0, 123.2, 120.1, 105.8, 105.6, 103.4, 98.0, 63.3, 61.0, 60.9, 56.0, 54.1, 54.0, 53.7, 50.5, 45.7, 44.2, 44.1, 43.7, 14.2. IR (KBr) cm^{-1}; 1700, 1411, 1314, 1217, 1111, 1002. FABMS, m/e 636 ($M^+ + H$); FABHRMS, m/e 636.2665 ($C_{32}H_{37}N_5O_9 + H^+$ requires 636.2669)

J. Am. Chem. Soc. v 116
p. 4171 Asai